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Capstone Approval Page

Capstone Title (print or type)

Isolation and analysis of *Arabidopsis thaliana* lines bearing *DRG* knock-out mutations and overexpressing GFP protein fusions: Are wild-type *DRG*s necessary for heat stress granule formation?

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HONORS THESIS ABSTRACT
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Findings: The genetic composition of *DRG* genes and GFPs does have an effect on the phenotype of *Arabidopsis*. Conversely, the presence of a wild type DRG gene fused to GFP (DRG1-GFP or DRG2-GFP) does not complement the non-functional *DRG* genes.

NORTHERN ILLINOIS UNIVERSITY

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Are wild-type *DRG*s necessary for heat stress granule formation?

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In Partial Fulfillment of the

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Department Of

Biological Sciences

By

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Contents

Abstract

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Introduction

DRG genes encode a highly conserved group of regulatory proteins that occur in the genomes of all eukaryotes. Such conservation suggests that they have a key role as physiological regulators in cell development (Ishiwawa, 2005). *DRG*s are classified as GTP-binding proteins that are involved in cell growth and differentiation, among other numerous biological processes, through the binding of GTP and GDP. Despite the suggested significance of *DRG*s, little is known about their specific function (Devitt, 1999). The purpose of this study is to analyze the role of *DRG*s in *Arabidopsis thaliana*, referred to as *DRG1* and *DRG2*, to assess their significance in cell development.

Approaches used to determine the function of *DRG*s included examining when and how the genes are expressed, observing protein accumulation and where it occurs, and if any changes take place in these characteristics in response to environmental stress. It is known that *DRG1* and *DRG2* respond to heat stress by forming aggregates referred to as heat stress granules (HSG), formations that are believed to be a protective measure to conserve mRNA and the future synthesis of the proteins (Wang, 2004).

Green fluorescent proteins, or GFP, can be used as a marker by associating with *DRG*s and revealing their location within the cell using a laser-scanning confocal microscope (Chalfie, 2006). It was found that *DRG1*-GFP and *DRG2*-GFP fusion proteins normally are dispersed in the cytoplasm. Following heat stress, fluorescence aggregates into large granules believed to be HSGs. One way to examine *DRG* function is through the use of *DRG*-GFP fusion proteins combined with loss-of-function *DRG* mutants (*drg1* and *drg2*). This has led to the objective of determining if *DRGs* are components of HSGs and whether HSG can form if *DRG1*, *DRG2*, or both genes are nonfunctional as a result of mutation. Due to the fact that GFP only exists in association with wild-type *DRG*, the inquiry has also been raised regarding whether the wild-type *DRG* present in the fusion is sufficiently active to complement the nonfunctional *DRG* mutations. The goal of this project has been to determine whether HSGs can still form in response to heat stress if one or both of the *DRG* genes is mutated, and if GFP can compensate for nonfunctional *DRGs* by allowing for HSG formation.

Materials and Methods

Genetic Crosses of DRG mutants and DRG-GFP Lines		
	L20, DRG1-GFP: $\frac{GFP1}{GFP1}$	**L21, DRG2-GFP: $\frac{GFP2}{GFP2}$**
L1: $\frac{drg1}{drg1}$	Line A: $\frac{drg1}{drg1}, \frac{GFP1}{GFP1}$	Line B: $\frac{drg1}{drg1}, \frac{GFP2}{GFP2}$
L2: $\frac{drg2}{drg2}$	Line C: $\frac{drg2}{drg2}, \frac{GFP1}{GFP1}$	Line D: $\frac{drg2}{drg2}, \frac{GFP2}{GFP2}$
L3: $\frac{drg1}{drg1}, \frac{drg2}{drg2}$	Line M: $\frac{drg1}{drg1}, \frac{drg2}{drg2}, \frac{GFP1}{GFP1}$	Line N: $\frac{drg1}{drg1}, \frac{drg2}{drg2}, \frac{GFP2}{GFP2}$

Table 1: **Genetic Crosses and Resulting Plant Line Genotypes.** Genetic crosses of DRG mutants and DRG-GFP Lines resulting in plant lines A, B, C, D, M and N that were the focus of this study, the ultimate goal being to obtain plant lines with the above genetic composition in order to test phenotypes with verifiable phenotypes.

Arabidopsis thaliana plants were grown in soil in a growth chamber under a 10-hour light/14-hour dark photoperiod at 20 degrees Celsius. Rosette leaves were collected from 1-week old and 5-week old plants. Root tips were collected from plants grown on Murashige-Skoog (MS) plates that were grown for 6 days and incubated for 1 week under constant light at 24 degrees Celsius.

The Edwards DNA preparation technique was used to prepare leaf tissue for use in the polymerase chain reaction (PCR). Leaf tissue was transferred to 1.5 ml microfuge tube. The tissue was ground using a Kontes pestle. Two installments of Edwards buffer were added to the tube to equal 400 µl of buffer. The tube was placed in a centrifuge to vortex the DNA into a pellet. Three hundred microliters of isopropanol was added to a fresh microfuge tube, and 300 µl of the supernatant from the initial tube was transferred to the fresh tube. The mixture of supernatant and isopropanol was pelleted for 5 minutes in the centrifuge. The supernatant was poured out of the tube, which was then inverted to allow any remaining supernatant to drain out of the tube. The tube was allowed to air dry. The pellet that remained in the tube was re-suspended in 1 ml of water and centrifuged for 2 minutes to pellet any remaining debris. The resulting supernatant was stored at -20 degrees Celsius for use in PCR.

PCR was performed to amplify DNA through thermal cycling in order to facilitate DNA replication. A typical program of cyclic steps was as follows (˚Celsius/minute): 94˚/ 2, [94˚/0.5, 50˚/0.5, 72˚/1.5] x 40 cycles, 75˚/5, 4˚/hold.

DNA was analyzed using gel electrophoresis based on the size of separated fragments that migrated across the gel due to the presence of an electric field.

Plant lines of known genetic composition were grown on MS plates and subjected to a root growth assay. The roots of each plant were measured to analyze the relationship between genetic composition and physical growth.

Results

Seeds from all 6 plant lines were planted and grown in soil and on MS plates to provide material for testing in the subsequent experiments. The original set of plants constitutes the parent plants, and the seeds from those plants were collected and grown to comprise the offspring plants referred to in the following experiments. Screening a population of plants refers to a sample of plants from each line being tested to serve as representative of the entire population of plants from each line. Individual plants within the offspring population of each line were tested in order to analyze the segregation of genes from the parent plants using a statistical analysis. Such analysis was done in order to verify the genetic composition of the parent plants and confirm the genotype of the individual plants. This type of analysis details that individual offspring plants comprised of the same homozygous genotype can verify that the parent plants are homozygous for that genotype, whereas individual offspring plants confirmed to vary between multiple genotypes verifies that the parent plants are heterozygous and that the alleles segregated within the offspring.

The parent plants from the populations of Lines A, B, C, D, M and N were tested for the presence of GFP1 wild type, GFP1 insertion, GFP2 wild type, and GFP2 insertion. The plants were tested using the primers 10-510/10-511 for GFP1 wild type, 02-339/10-511 for GFP1 insertion, 10-512/10-513 for GFP2 wild type, and 02-339/10-513 for GFP2 insertion. The results for the experiment are shown in Figure 1 and summarized in Table 2. It was expected that Lines A, C, and M would contain GFP1 insertion indicated by a band at 600 base pairs and GFP2 wild type indicated by a band at 600 base pairs, and that Lines B, D, and N would contain GFP2 insertion signified by a band at 500 base pairs and GFP1 wild type signified by a band at 800 base pairs. The more distinct bands found in the lanes for GPF1 insertion is believed to be an artifact due to the bands not being at the expected base pair size, therefore the results for that gene are inconclusive. The faint band found present for GFP2 wild type in Line N is not believed to be indicative of GFP1 wild type presence due to the robust bands found in Lines B, C, and M. The faint bands for GFP2 insertion in Lines A, C and M are not believed to be indicative of GFP2 presence due to their contrast with the robust bands found in Lines B, D, and N.

The parent plants from the populations of Lines A, B, C, D, M and N were tested for the presence of *DRG1*, *DRG2*, *drg1* and *drg2*. The plants were tested using the primers 10-724/10-723 for *DRG1*, 10-724/10-378 for *drg1*, 10-725/10-726 for *DRG2*, and 10-725/10-378 for *drg2*. The results for the experiment are shown in Figure 2, confirming that the genotypes for all six lines correspond to the genotypes listed in Table 1. It was expected that Lines A and B would contain *drg1* indicated by a band at 730 base pairs and *DRG2* indicated by a band at 952 base pairs; Lines C and D would contain *drg2* signified by a band at 750 base pairs and *DRG1* signified by a band at 824 base pairs; and Lines M and N would contain both *drg1*indicated by a band at 730 base pairs and *drg2* signified by a band at 750 base pairs. The bands seen at sizes other than at the expected base pair size are artifacts and are therefore disregarded as indicating presence of the genes. The results of Figure 2 are summarized in Table 2.

Individual plants from the populations of Lines A, B, C, D, M and N were tested for the presence of GFP1 wild type, GFP1 insertion, GFP2 wild type and GFP2 insertion. The plants were tested using the primers previously mentioned. The results for the

experiment are shown in Figure 3 and summarized in Table 3. It was anticipated that Lines A, C, and M would contain GFP1 insertion and GFP2 wild type; it was anticipated that Lines B, D, and N would contain GFP2 insertion and GFP1 wild type. It was found that Plant A was consistent with expectancies for the desired genotype but not with Figure 1 because Line A did not work in that experiment. Plant B was consistent with expectations and Figure 1; it was found to be heterozygous for GFP2 and segregating in the offspring plants. Plant C was consistent with expectations and Figure 1, but bands present in GFP2 insertion suggest false positives or heterozygosity. Plant D was consistent with expectations and Figure 1 with the exception of GFP1 wild type in D4 plant that could be a false negative or some other error. Plant M was consistent with expectations and Figure 1 for GFP2, but there were erroneous results for GFP1 because no bands in either wild type or insertion were observed, indicating possible false negatives. Plant N was consistent with expectations and Figure 1.

Individual plants from the populations of Lines A, B, C, D, M and N were tested for the presence of *DRG1*, *drg1*, *DRG2* and *drg2*. The plants were tested using the primers previously mentioned. The results for the experiment are shown in Figure 4 and summarized in Table 3. According to the results in Figure 4, the genotypes for all six lines were confirmed to correspond to the genotypes represented in Table 1. It was anticipated that Lines A and B contain *drg1* and *DRG2*; Lines C and D contain *drg2* and *DRG1*; Lines M and N contain both *drg1* and *drg2*. Plants B, D and M were consistent with expectations and Figure 2. Plant A was consistent with expectations and Figure 2 with the exception of plant A2 for *drg1*, suggesting a possible false negative. Plant C was consistent with expectations and Figure 2 for *DRG1*, but it was observed to be heterozygous and segregating for *DRG2* and *drg2*. Plant N was consistent with expectations and Figure 2 with the exception of plant N2 for *drg1*, suggesting a possible false negative.

Plants from lines A, B, C, D, M and N were grown on MS plates and subjected to a root growth assay. The roots of each plant line were measured and compared to plants from Lines 1 (single mutant for *drg1*), 2 (single mutant for *drg2*), 3 (double mutant for *drg1,drg2*), 17 (wild type), 20 (GFP1 insertion) and 21 (GFP2) that served as controls to analyze the effects of DRG genotype composition and root growth, the results of which are depicted in Figure 5. All six plant lines exhibited similar growth to the control lines represented by Lines 1, 2 and 3 that did not contain GFP. The only plant lines to show significant difference in root growth were the double-mutant plants from Lines 3, M and N, all three double-mutant plants being equivalent in growth. This suggests that the *DRGs* compensate for one another when one is nonfunctional, but when both are knocked out, the plant displays developmental abnormalities. This finding also suggests that GFP presence in Lines M and N does not compensate for *DRG* mutations due to similar growth to Line 3 that does not contain GFP. The average root lengths and standard deviation among all the plants in each line were assessed and are represented in Figure 6. Correlations could be observed among certain plant lines. Control Lines 1, 2, 17, along with experimental Lines A, B and D all exhibited similar root lengths that are approximately 27 mm; similarly, control Lines 20, 21 and experimental Line C showed similar root lengths above 33 mm; control Line 3 and experimental Lines M and N all expressed root lengths that were approximately 13 mm.

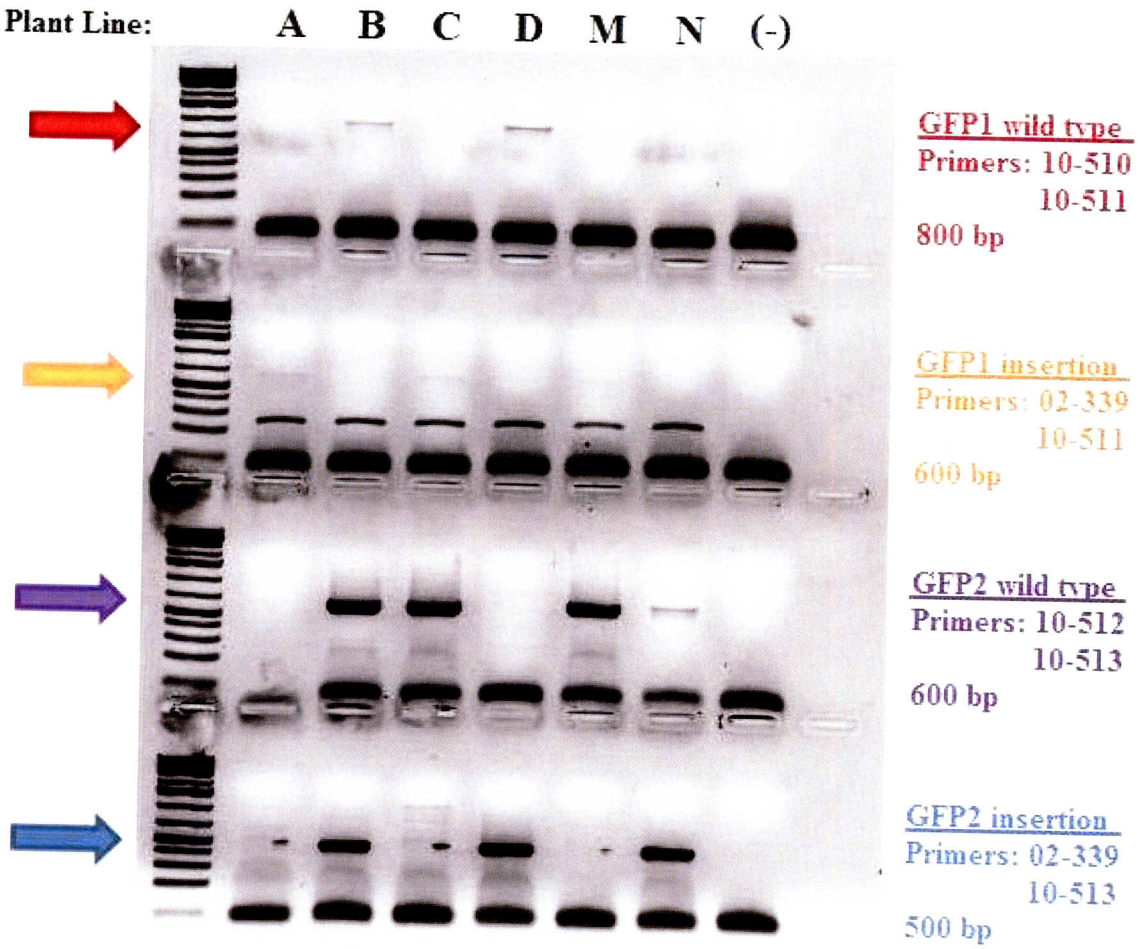

Figure 1: Parent Population Screen for GFP Composition. The parent plants from the populations of Lines A, B, C, D, M and N were tested for the presence of GFP1 wild type, GFP1 insertion, GFP2 wild type, and GFP2 insertion. The arrows in the figure represent the bands of interest at the expected base pair size if the genes of interest are present, and the bands seen at sizes other than at the expected base pair size are artifacts and are therefore disregarded as indicating presence of the genes.

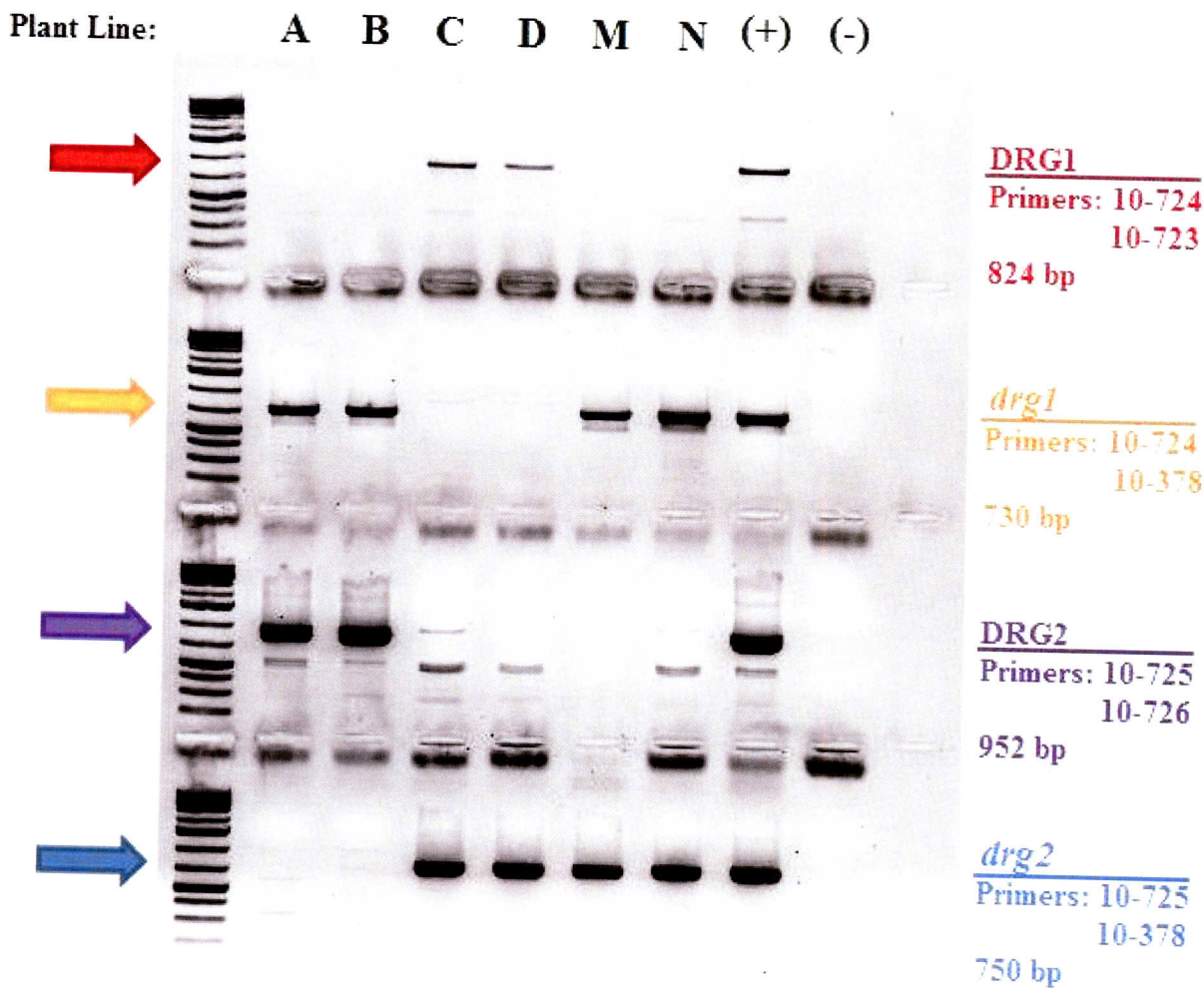

Figure 2: Parent Population Screen for DRG Composition. The parent plants from the populations of Lines A, B, C, D, M and N were tested for the presence of *DRG1*, *DRG2*, *drg1* and *drg2*. The results verified that the genotypes for all six lines correspond to the genotypes listed in Table 1. The arrows in the figure represent the bands of interest at the expected base pair size.

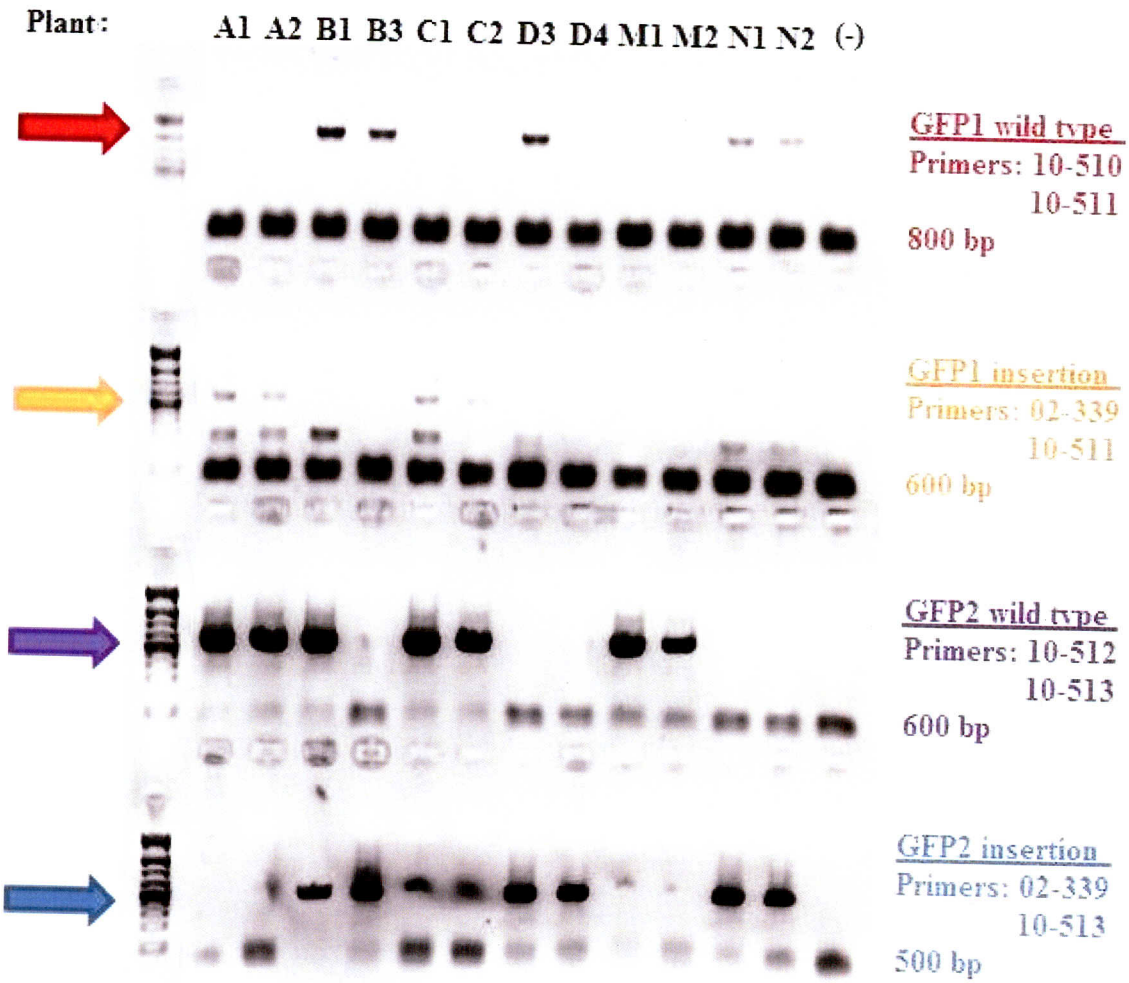

Figure 3: Individual Plant Screen of GFP Composition. Individual plants from the populations of Lines A, B, C, D, M and N were tested for the presence of GFP1 wild type, GFP1 insertion, GFP2 wild type and GFP2 insertion. The arrows in the figure represent the bands of interest at the expected base pair size.

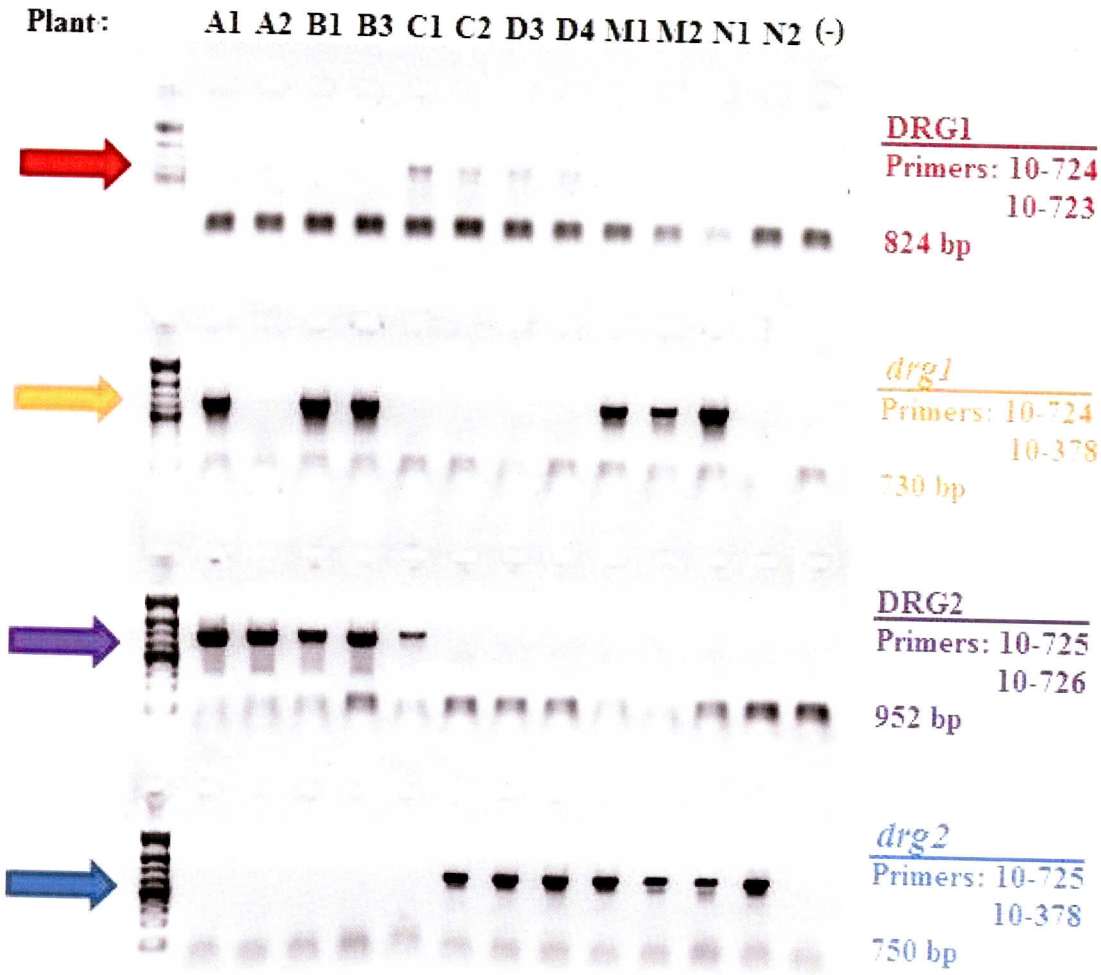

Figure 4: Individual Plant Screen of DRG Composition. Individual plants from the populations of Lines A, B, C, D, M and N were tested for the presence of *DRG1*, *drg1*, *DRG2* and *drg2*. The arrows in the figure represent the bands of interest at the expected base pair size. According to the results, the genotypes for all six lines were confirmed to correspond to the genotypes represented in Table 1.

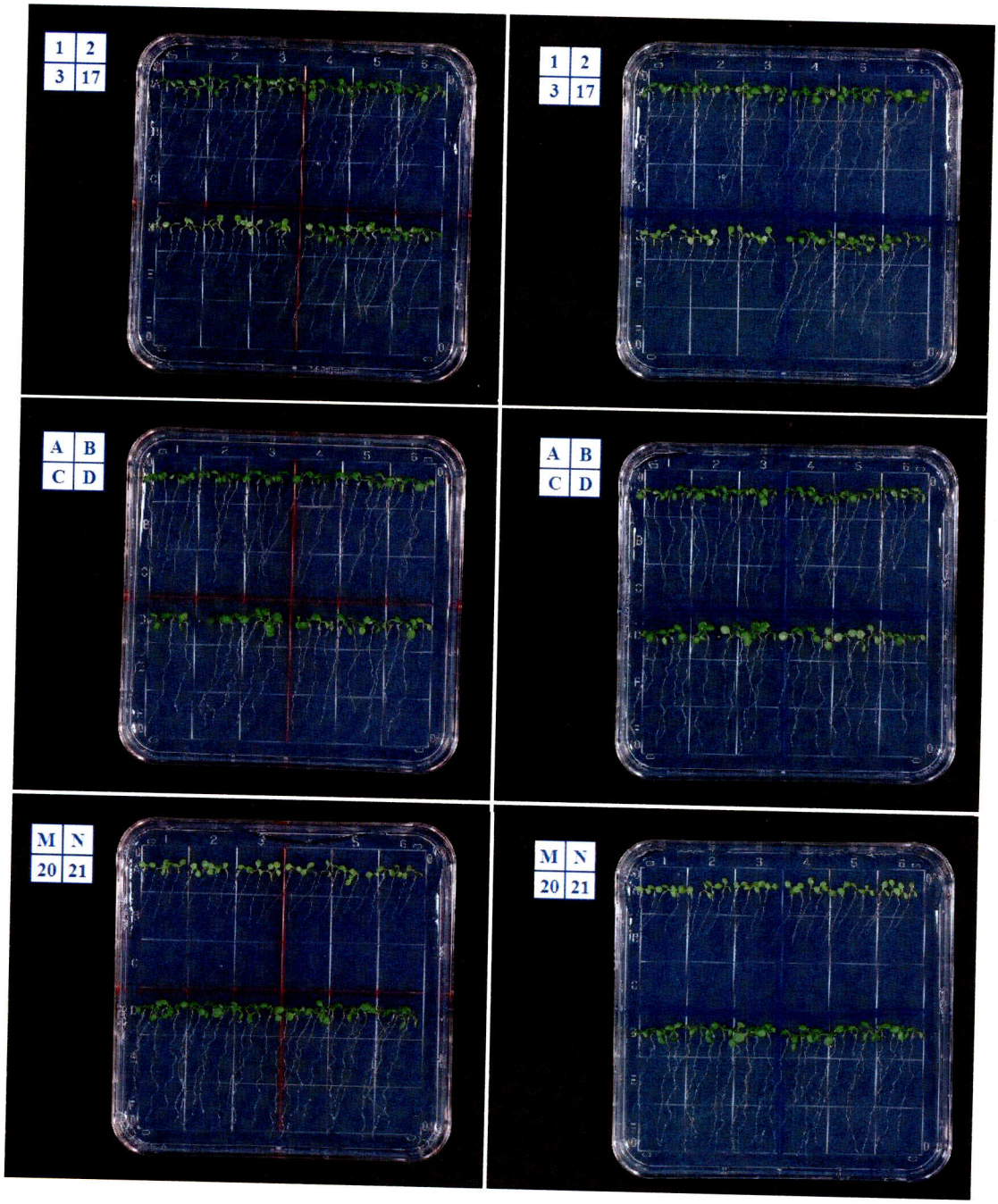

Figure 5: **Root Growth Assay.** Plants from lines A, B, C, D, M and N were grown on MS plates and compared to plants from Lines 1 (single mutant for *drg1*), 2 (single mutant for *drg2*), 3 (double mutant for *drg1,drg2*), 17 (wild type), 20 (GFP1 insertion) and 21 (GFP2) that served as controls to analyze the effects of DRG genotype composition and root growth. All six plant lines exhibited similar growth to the control lines represented by Lines 1, 2 and 3 that did not contain GFP.

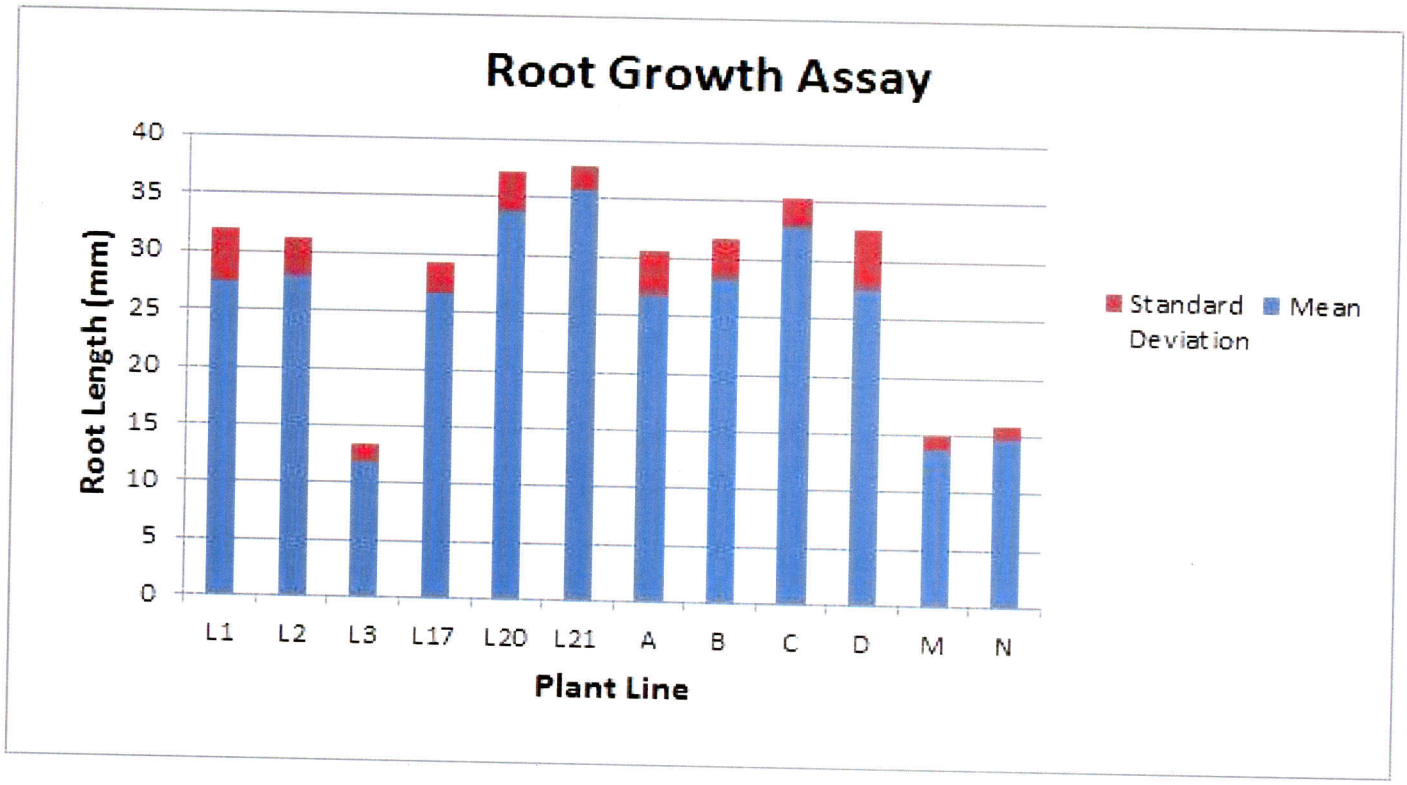

Figure 6: **Statistical Analysis of Root Growth Assay.** The average root lengths and standard deviation among all the plants in each line were assessed and are represented in Figure 6. Correlations could be observed among certain plant lines. Control Lines 1, 2, 17, along with experimental Lines A, B and D all exhibited similar root lengths that are approximately 27 mm; control Lines 20, 21 and experimental Line C showed similar root lengths above 33 mm; control Line 3 and experimental Lines M and N all expressed root lengths that were approximately 13 mm.

Genotype Compositions of Plants from Lines A, B, C, D, M and N									
Plant	**DRG1**	**drg1**	**DRG2**	**drg2**	**GFP1 wt.**	**GFP1 insert**	**GFP2 wt.**	**GFP2 insert**	**Analysis**
A	-	+	+	-	-	+	-	-	**P**
B	-	+	+	-	+	-	+	+	**A**
C	+	-	-	+	-	+	+	-	**S**
D	+	-	-	+	+	-	-	+	**S**
M	-	+	-	+	-	+	+	-	**S**
N	-	+	-	+	+	-	-	+	**S**

Table 2: Genetic Composition of Plant Line Populations. The genetic compositions of plants from the populations of Lines A, B, C, D, M and N were tested for the presence of *DRG1*, *drg1*, *DRG2*, *drg2*, GFP1 wild type, GFP1 insertion, GFP2 wild type and GFP2 insertion. The plants were analyzed on the basis of being satisfactory (S) for possessing the desired homozygous genotype, acceptable (A) for being confirmed as heterozygous, and poor (P) for the presence of erroneous results. The S designation means that the plant is ready to be utilized in future experiments for further analysis. The A designation signifies that the plant requires further crossing in order to achieve the desired homozygous genotype in order to be used for analysis. The P designation indicates that the plant requires further testing to determine the source of error in the experiment in order to conclude what needs to be done to obtain the desired genotype.

Genotype Composition of Individual Plants from Lines A, B, C, D, M and N									
Plant	DRG1	drg1	DRG2	drg2	GFP1 wt.	GFP1 insert	GFP2 wt.	GFP2 insert	Analysis
A1	-	+	+	-	-	+	+	-	S
A2	-	-	+	-	-	+	+	-	P
B1	-	+	+	-	+	-	+	+	A
B3	-	+	+	-	+	-	-	+	S
C1	+	-	+	-	-	+	+	+	P
C2	+	-	-	+	-	+	+	+	A
D3	+	-	-	+	+	-	-	+	S
D4	+	-	-	+	-	-	-	+	P
M1	-	+	-	+	-	-	+	-	P
M2	-	+	-	+	-	-	+	-	P
N1	-	+	-	+	+	-	-	+	S
N2	-	-	-	+	+	-	-	+	P

Table 3: Genetic Composition of Individual Plants. The genetic compositions of individual plants from the populations of Lines A, B, C, D, M and N were tested for the presence of *DRG1*, *drg1*, *DRG2*, *drg2*, GFP1 wild type, GFP1 insertion, GFP2 wild type and GFP2 insertion. The plants were analyzed on the basis of being satisfactory (S) for possessing the desired homozygous genotype, acceptable (A) for being confirmed as heterozygous, and poor (P) for the presence of erroneous results.

Symbol	Interpretation
+	Present, Positive Result
-	Absent, Negative Result

Conclusion

The genetic composition of *DRG* and GFPs does have an effect on the phenotype of *Arabidopsis*, as observed in the root growth assay. As seen in Figure 5, only plants that possess both nonfunctional *DRG1* and *DRG2* genes exhibit irregular growth, suggesting that the *DRGs* have a codependent role in which the composition of only one *DRG* mutant will result in the other gene compensating in plant development, but loss of functionality in both *DRGs* renders the plant developmentally-compromised.

This study has also led to the conclusion that the presence of a wild type *DRG* fused to GFP (*DRG1*-GFP or *DRG2*-GFP) in Lines M and N, respectively, does not complement the non-functional *DRG* genes. The fusion of GFP to wild-type *DRG* in conjunction with double-mutant *DRGs* in Lines M and N could exhibit two possible phenotypes that would propose whether GFP is able to complement the double mutation. The double-mutant phenotype without the presence of GFP fusion as it is observed in Line 3 exhibits a phenotype that comprises stunted root growth and yellow coloring, whereas the phenotype observed in plants that are heterozygous for *DRG*/*drg* exhibit the same phenotype observed in wild-type plants. Due to the fact that Lines M and N possess double mutations like that of Line 3 but also contain wild-type *DRG* in the presence of the *DRG*-GFP fusion, these lines could theoretically exhibit either phenotype. The conclusion that GFP does not complement nonfunctional *DRG* has been drawn based on the results of this study that reveal the plant lines possessing double mutations for *DRG1* and *DRG2* along with GFP (Lines M and N) have identical stunted growth in comparison to the double-mutant plant that does not contain GFP (Line 3).

The next step was to subject the experimental plant lines to heat stress and examine the root tips under a confocal microscope. A preliminary experiment utilizing confocal microscopy was observed. In this analysis, plant Lines 20 ($\frac{GFP1}{GFP1}$), 21 ($\frac{GFP2}{GFP2}$), M ($\frac{drg1}{drg1}$, $\frac{drg2}{drg2}$, $\frac{GFP1}{GFP1}$) and N ($\frac{drg1}{drg1}$, $\frac{drg2}{drg2}$, $\frac{GFP2}{GFP2}$) were subjected to heat stress and their root tips examined using confocal microscopy. It was observed that plants from Lines 20 and 21 exhibited formation of HSGs as expected. In contrast, no plants that expressed GFP in their root tips from Line M exhibited HSGs, and only one plant from Line N displayed such aggregation. This has led to the preliminary conclusion that GFPs do not compensate for nonfunctional *DRG* mutants in the aggregation of HSGs. Being an initial investigation, further plant testing will be conducted with the fundamental goal being to uncover the role of *DRG* and GFP as factors in plant development and as a protective mechanism in response to heat stress in *Arabidopsis*.

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